Screening of Tyrosinase Inhibitor, Antioxidant and Cytotoxicity of Dried Sea Cucumber from Tomini Bay, Indonesia

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ABSTRACT

Background: Indonesia, as a tropical country, is one of the important producers of sea cucumbers (beche-de-mer). Sea cucumber is a marine invertebrate that contains attractive bioactive secondary metabolites and these metabolites can be used for health as well as cosmetics. Objective: The aim of the study was to determine the activity of tyrosinase inhibitors, antioxidants, and cytotoxicity of sea cucumber methanolic extract. Methods: Dried sea cucumber samples were taken from Boalemo waters, Tomini Bay, Indonesia. Tyrosinase inhibitor assay was carried out spectrophotometrically using tyrosinase enzymes and L-DOPA as a substrate and antioxidant tests were carried out by DPPH method. Cytotoxicity test against human breast cancer cell line (T47D) was conducted using the MTT assay. Results: The study showed that Bohadschia vitiensis had the best tyrosinase inhibitor activity with IC₅₀ value of 0.28 mg/ml. The DPPH free radical scavenging testing showed that all sea cucumbers had weak antioxidant activity. On the other hand, cytotoxicity assay revealed that several sea cucumbers had good cytotoxicity against T47D cells, where Holothuria atra and Bohadschia marmorata showed strong cytotoxicities with IC₅₀ values of 23.0 and 28.1 μg/mL, respectively. Conclusion: Based on the study, it can be concluded that the dried sea cucumber from the Tomini bay region, Indonesia, has the potential to be developed as a source of tyrosinase inhibitors and cytotoxic agents.

Key words: Sea cucumber, Screening, Tyrosinase inhibitor, Antioxidant, Cytotoxicity.

INTRODUCTION

Sea cucumber is a group of animals that lives in the sea. Sea cucumbers are usually soft-bodied echinoderms, looking like a cucumber, elongated, worm-like organisms, with a leathery skin and gelatinous body.¹ They included in the Holothuroidae class and Echinodermata phylum. Sea cucumbers have long been fished and exported as dried. The dried form of sea cucumbers is called beche-de-mer in the Pacific and the Indian Ocean, balat in parts of Malaysia and the Philippines and in Indonesia popular as teripang.² Sea cucumber has long been used by the people of Asia and the Middle East as food and traditional medicine.³ Teripang has a rich of nutrients, such as vitamin A, vitamin B1, vitamin B2, vitamin B3, and minerals, especially calcium, magnesium, iron, and zinc. It’s also known to have broad spectrum of biological and pharmacological activity, such as anti-inflammatory i.e Holothuria edulis, cytoprotective i.e Holothuria atra, neuroprotective i.e Stichopus japonicus and cytotoxicity.⁴⁻⁶

Recently, research to explore marine bioactive compounds especially marine medicines and cosmetics shows increasing trend due to the diversity of the chemical structures and their bioactivities.⁷ In the period of 1965 - 2014, around 25.700 new bioactive compounds were successfully identified from the marine environments.⁸ Sea cucumber as a source of potential bioactive compounds from the marine is currently being developed as a cosmetics material. This is related to the content of collagen, fucoidan, and phenolic compounds that found in sea cucumbers.⁹⁻¹² Aside from being a cosmetics ingredient, biodiscovery of anticancer compounds from sea cucumber is also carried out by many researchers.¹³ Sea cucumbers have attractive anticancer properties because they contain high saponin compounds. Some cytotoxic saponins found in sea cucumbers are holothurin, frondoside, and echinoside.¹³⁻¹⁵

Boalemo District that located at Gorontalo Province-Indonesia, is one of the important sea cucumber fisheries sources. Sea cucumbers from this area come from fishermen who catch it from the Tomini Bay waters. In general, sea cucumbers after being caught then removed the contents of the viscera, boiled, then dried using sunlight. So far, the information about biopotency of dried sea cucumbers from this region is still limited. This study aimed to investigate the activity of tyrosinase inhibitor, anti-
MATERIALS AND METHODS

Samples Preparation and Extraction

Dried sea cucumbers were collected from Tilamuta village, Boalemo District, Gorontalo-Indonesia. Samples used in this study were dried sea cucumbers that processed by a fisherman in the village. Sea cucumbers were taken from around waters of Tomini Bay. The samples obtained were identified based on Purcell16 and Setyastuti and Purwati.1 The extraction process was conducted by maceration using methanol for 12 h (3 times). The extract was filtered using filter paper and evaporated in vacuum rotavapor. The methanol was subsequently dried in vacuum concentrator until dry extract obtained.

Tyrosinase Inhibitor Assay

Tyrosinase inhibition assay was conducted according to Zamani et al.17 The sample was dissolved with DMSO as a stock solution. A range of extract concentration was prepared by dissolving concentrated extract using phosphate buffer (pH 6.5). A total of 70 µL of extract solution and 30 µL tyrosinase enzyme (Sigma, 333 units of mL−1 in phosphate buffer solution) were put into 96-well plate and incubated for 5 min. The mixture then was added with 110 µL of substrate (L-DOPA 12 mM) and incubated at 37°C for 30 min. The absorbance was measured at a wavelength of 492 nm using Microplate Reader. Kojic acid was used as a positive control. IC50 values were determined by using Minitab statistic package version 16.0.

Antioxidant Assay

The antioxidant assay was conducted according to Batubara et al.18 The dried extracts were dissolved in methanol and prepared in a range of concentrations as a sample solution. A total of 100 µL of the sample solution and 100 µL of 125 µM DPPH solution were put into a 96-well plate. Samples were incubated at room temperature for 30 min. After that, the absorbance was measured at a wavelength of 517 nm using a microplate reader. Ascorbic acid was used as a positive control. Probit analysis to calculated IC50 value was carried out by using Minitab version 16.0.

Cytotoxicity Assay

Human breast ductal carcinoma cell line (T47D) was routinely grown and maintained in RPMI medium with 10% FBS and 1% penicillin-streptomycin. The cell line was incubated in a moisture-saturated atmosphere containing 5% CO2 at 37°C. Cytotoxicity test was conducted according to Nursid et al.19 To each well of the 96-well microplate containing 100 µL of cell suspension (1.5 × 104 cells) was added 100 µL of extracts with a concentration of 5, 25, 125, and 625 µg/mL then the plate was incubated in a CO2 for 24 h. After the addition of 100 µL of 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide saline solution (0.5 mg/mL) to each well of a microplate reader, the plate was then incubated for 4 h under the same conditions in the CO2 incubator. After incubation, the optical density was measured at 570 nm in a microplate reader (Thermo Scientific). Morphology cell was analyzed by using an inverted microscope. The IC50 values were defined as the concentrations of a compound which inhibited 50% of the cell growth. IC50 value was determined by using Minitab probit analysis version 16.0.

RESULTS

In this study, 15 species of dried sea cucumbers were collected. After all samples were extracted with methanol, the extract was tested as a tyrosinase inhibitor, antioxidant, and cytotoxicity (Table 1).

The yield of sea cucumber extract ranged between 2.5-10.5 %. The result of inhibitor tyrosinase assay showed that Bohadschia vitiensis, B. argus, and Stichopus hermanii had the best activity with IC50 values of 0.28; 1.61; and 1.65 mg/mL. Antioxidant activity testing showed that the Holothuria edulis, H. scabra and B. vitiensis had the best activity with IC50 of 1.78; 4.1; and 7.19 mg/mL, respectively. While MTT test showed that Holothuria atra, and B. marmorata had the strongest cytotoxicity against T47D cells with IC50 values of 23.0 and 28.1 µg/mL. Some species of sea cucumber i.e Holothuria edulis, Bohadschia ocellata, Pearsonothuria graeffei, and Bohadschia vitiensis have high cytotoxicity with IC50 values ranging from 54.2 - 74.7 µg/mL. Morphology features of the T47D cells morphology were still seen intact, it was similar with the untreated cell (cell control), but at doses of 25 µg/mL and 125 µg/mL, the cells morphology changed to be unclear and the cell lost its integrity. Tyrosinase inhibitor profile, antioxidant activity, and cytotoxicity of various dried sea cucumber extracts were presented in Figure 2.

DISCUSSION

Brown skin on people in the tropics is often a concern in the field of beauty. Recently, many studies were conducted to explore materials or compounds that can act as a whitening agent. The brown color of the skin occurs due to excessive production of melanin. Melanin is responsible for human skin color. Tyrosinase is a metalloenzyme containing copper which is widely distributed in nature, including bacteria, fungi, plants, and animals. In mammals, tyrosinase plays a role in pigmentation of the skin, eyes, and hair.20 Tyrosinase is involved in the pigmentation process, by changing the L-tyrosine substrate to L-DOPA and converting the L-DOPA substrate

<table>
<thead>
<tr>
<th>No</th>
<th>Species</th>
<th>Tyrosinase inhibitor (µg/ml)</th>
<th>DPPH (mg/ml)</th>
<th>Cytotoxicity (µg/ml)</th>
</tr>
</thead>
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<td>1</td>
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<td>2</td>
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<td>≥500</td>
<td>159.86</td>
<td>68.90</td>
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<td>4</td>
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<td>919.68</td>
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<td>54.21</td>
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<tr>
<td>6</td>
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<td>15.64</td>
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<td>Stichopus hermanii</td>
<td>1.9</td>
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<td>&gt; 10,000</td>
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<tr>
<td>16</td>
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<td>18</td>
<td>Doxorubicin</td>
<td>-</td>
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The high reactivity of DOPA or dopaquinone causes polymerization reactions to form melanin (brown pigment). The mechanism of melanin formation through catalyzed tyrosine by tyrosinase enzyme to be 3,4-dihydroyphenylalanine (DOPA), then DOPA is oxidized to dopaquinone and then reactions that produce eumelanin or pheomelanin pigments. Skin pigmentation can be reduced by inhibiting the activity of tyrosinase enzymes thereby inhibiting the reaction of L-tyrosine to L-DOPA or called inhibition of monophenolase and inhibiting the reaction of L-DOPA to dopaquinone or inhibition of diphenolase. In melanin formation reactions often produce reactive oxygen species (SOR) which can accelerate skin pigmentation. The enzyme tyrosinase and substrate reactions produce a brown color. Compounds that can inhibit the process of melanogenesis are expected to be applied in the cosmetic industry as whitening agents. Tyrosinase inhibitor makes melanin production reduced because the activity of this enzyme is the rate-controlling step of melanin synthesis. More specifically, tyrosinase inhibitors can be used in dermatological treatments for skin whitening by inhibiting the enzymatic process of melanin formation.

Husni et al. found that sand sea cucumber extract can inhibit the activity of the tyrosinase enzyme, while Yoon et al. and Lee et al. states that sea cucumbers have the potential as inhibitors of melanogenesis. In this study, B. vitiensis had the best tyrosinase inhibitory activity with an IC₅₀ value of 0.28 mg/ml even though the activity was not as good ascorbic acid (IC₅₀ of 0.19 mg/ml). The antioxidant activity of B. vitiensis probably increases if the active compound is tested in the form fractions or a single compound. Possible compounds that have tyrosinase inhibitor activity from sea cucumbers are from phenolic compounds.

Research to explore of active compounds from the sea other than as a whitening agent is also carried out to find compounds that can inhibit or neutralize free radicals. Reactive oxygen species (ROS) can cause damage to biomolecules such as proteins, fats, enzymes, DNA or RNA. Bio-molecular damage due to ROS correlates strongly with the process of carcinogenesis, mutagenesis, and cancer occurrence. Sea cucumber extract activity related to antioxidant properties were revealed by Banderanayake and Des Roches who found the activity of Holothuria atra as an ultraviolet protector. Ghanbari found that protein hydrolyzate from Actinopyga lecanora sea cucumber can inhibit DPPH and Ferrous ion-chelating (FIC) free radical. In addition, antioxidant properties of Stichopus japonicus was revealed by Wang et al. In this study, the antioxidant activity of the sea cucumbers tested was included in the weak category in compare with an ascorbic acid activity which had an IC₅₀ value of 0.00748 mg/ml. Based on this, it can be said that the antioxidant activity of the dried sea cucumber methanol extract cannot be determined well by the DPPH method.

The results of the cytotoxicity test showed that more extracts had good activity in contrast with tyrosinase inhibitor and antioxidant tests. There were six sea cucumber extracts which have good cytotoxicity where the best potential was found in H. atra (Table 1). Many studies reported that the cytotoxicity of sea cucumbers is related to saponin (triterpenes glycosides) compounds found in sea cucumbers. Saponins in sea cucumbers have similar structures with saponins found in terrestrial plants, namely.

These compounds were contained in the body wall and viscera. So far, approximately 150 types of saponins from the Holothuroidea sea cucumber have been identified. Some cytotoxic saponins from sea cucumbers that have been identified, for example, are frondoside, echinose, holothurin, and philinopside, each of which was isolated from sea cucumber Cucumaria frondosa, Pearsonothuria graeffei, Holothuria vagabunda, and Pentacta quadrangularis. Cytotoxic compounds from sea cucumber H. atra, desulfated echinose B (DEB), had IC₅₀ values against A459 cells (human lung cancer cell line), HuH-7 (hepatocarcinoma cell line), HepG2 (liver cancer cell line), and B16F10 (murine melanoma cell line) with a range between 0.5 - 2.5 µM. Experimental and computational studies showed that DEB can act as an inhibitor of the PAK1 oncogenic kinase.

CONCLUSION

Based on the screening inhibitor tyrosinase, antioxidant, and cytotoxicity activity, B. vitiensis showed the best tyrosinase inhibitor with IC₅₀ value of 0.26 mg/ml while the antioxidant test showed weak activity against DPPH free radicals. Sea cucumber with the strongest cytotoxicity was shown by H. atra and B. marmorata with IC₅₀ values of 23.0 µg/ml and 28.1 µg/ml.

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CONFLICT OF INTEREST

There are no conflicts of interest.

ABBREVIATIONS

DMSO: Dimethyl sulfoxide; L-DOPA: L-3,4-dihydroxyphenylalanine; DPPH: 2,2-diphenyl-1-picrylhydrazyl; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; T47D: Human breast ductal carcinoma cell; RPMI: Roswell park memorial institute medium; FBS: Fetal bovine serum.

REFERENCES